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Molecular and epidemiological population-based integrative analysis of human and animal *Mycobacterium bovis* infections in a low-prevalence setting

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ABSTRACT

Human *Mycobacterium bovis* infections are considered to be due to reactivations, when involve elderly people, or to recent transmissions, when exposure is occupational. We determined the cause of *M. bovis* infections by genotyping *M. bovis* isolates in a population-based study integrating human and animal databases. Among the 1,586 tuberculosis (TB) cases in Asturias, Northern Spain (1,080,000 inhabitants), 1567 corresponded to *M. tuberculosis* and 19 to *M. bovis*. The number of human isolates sharing genotype with cattle isolates was higher than expected (47%) for a setting with low prevalence of bovine TB and efficient control programs in cattle. The risk of exposure to infected animals was probable/possible in most of these matched cases (77.7%). Recent transmission was the likely explanation of most *M. bovis* infections in elderly people. A potential human-to-human transmission was found. Our study illustrates a model of collaboration between human and animal health professionals to provide a precise snapshot of the transmission of *M. bovis* in the human-animal interface.

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1. Introduction

Mycobacterium bovis is the main causative agent of tuberculosis (TB) in animals. This pathogen has a wide range of hosts (Shrikrishna et al., 2009), including humans, who become infected through ingestion of unpasteurized cow's milk or milk products, aerosol inhalation, or direct contact with mucous membranes and skin abrasions from infected animals. Most clinical cases of TB in humans due to *M. bovis* are attributed to recrudescence of long-standing latent infection contracted before widespread pasteurization of milk (Mechai et al., 2011), occupational exposure (Khattak et al., 2016), or infection contracted abroad (Harris et al., 2007). Person-to-person transmission has mainly been described among immunocompromised patients or in cases of very close contact (Etchechoury et al., 2010; Guerrero et al., 1997).

Transmission of *M. bovis* from cattle and goats to humans was once common in Spain, although this transmission has been markedly reduced by decades of disease control in cattle and by routine pasteurization of cow's milk. Today, a relatively small

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proportion (<2%) of the total number of human TB cases in Spain are caused by *M. bovis* (Rodriguez et al., 2009).

Despite the zoonotic character of this disease, exchange of information between human and animal health authorities has been hampered owing to the lack of common strategies. In this study, we aimed to perform a long-term (2006–2014) integrative analysis of human and animal infection by *M. bovis* in the population of Asturias (northern Spain), where the prevalence of bovine TB in cattle is much lower than the overall prevalence in the rest of Spain (0.21% *vs* 1.72% in 2014). In addition, we refined our study of the reasons behind each case of *M. bovis* infection to go beyond assignation of reactivation/recent transmission based on the age of the case and general epidemiological data. Our coordinated epidemiological research approach enabled us to perform a refined fingerprinting-based comparative analysis of human isolates and isolates from cattle from farms located in the patient's area of residence.

2. Material and methods

2.1. Selection of isolates and distribution of tasks

The human sample comprised all *M. bovis* isolates obtained from human cases in the population of Asturias during the study period (2006–14). The animal sample comprised at least one *M. bovis* isolate per farm sharing a spoligotype with any human case within the same health district (Asturias is divided into 8 health districts). In specific cases, samples from adjacent health districts were also included following criteria of proximity with the household of the case. All the cultures from human specimens and the identification and genotyping, by spoligotyping and RD (regions of difference) analysis, of mycobacteria isolated from humans were carried out in the laboratory in Asturias. All the cultures from animal specimens and the identification and genotyping of mycobacteria isolated from animals by spoligotyping were carried out in the laboratory in VISAVET. Variable-number-tandem-repeats (VNTR) analysis was done in the laboratory in Gregorio Marañón Hospital.

2.2. Spoligotyping and additional genotypic analysis

DNA was extracted using a simple boiling method. The spoligotyping protocol was performed as described by Kamerbeek et al. (1997). Spoligotype signatures were matched against the *M. bovis* spoligotype database (http://www.mbovis.org) and the global spoligotype database, SpolDB4. *M. tuberculosis* H37 Rv and *M. bovis* BCG were systematically included in each hybridization assay. For the human isolates RD analysis was conducted (Parsons et al., 2002) for RD1, RD4, RD9, RD10, RD12, RDpan, RD17, N-RD17, and N-RD25. *gyr*B sequence analysis was performed to identify *M. caprae* (Kasai et al., 2000).

2.3. MIRU-VNTR analysis

9-MIRU-VNTR analysis (QUB-11a, QUB-3232, ETR-A, QUB-11b, MIRU 4, MIRU 26, MIRU 31, QUB-26, MIRU 48) of human isolates was performed following a protocol described elsewhere (Navarro et al., 2014). When a higher discrimination was pursued, in the potential human-human matches, 24-MIRU-VNTR typing was performed as described by Navarro et al. (2011).

2.4. Comparative analysis between human and animal fingerprints

We applied a 3-step approach to screen for and confirm the presence of genotypes shared by human and cattle isolates. First, we screened farms to identify animals sharing a spoligotype with an infected person living in the same area using the Spanish Animal Mycobacteriosis Database, mycoDB.es. mycoDB.es is a restricted database for Veterinary Services and Laboratories involved in the National Bovine Tuberculosis Eradication Program (Rodriguez-Campos et al., 2012). This database includes most of the *Mycobacterium tuberculosis* complex (MTBC) isolates from different animal species (mainly cattle) from 1996 to 2016.

Second, isolates sharing spoligotypes from the previous step were analyzed using MIRU-VNTR based on 3 highly discriminatory loci (VNTR 3232 [QUB-3232], VNTR 2165 [ETR-A], and VNTR 2163a [QUB-11a]). If differences were found between the human and animal isolates (differences in 2 or more loci, or differences in more than 2 repetitions in 1 locus), isolates were considered different and the analysis was closed. If the isolates shared the same pattern for the 3 discriminatory loci, we extended the analysis to complete 9-MIRU-VNTR in order to finally confirm identity or similarity (single locus variations: differences in one repetition in only one locus) or rule it out.

2.5. Epidemiological survey

The medical records of patients with TB (causative agents *M. tuberculosis* and *M. bovis*) included in the study were reviewed retrospectively. The risk of exposure to potentially infected animals was classified as follows:

- Probable: highly probable occupational exposure (agricultural workers or rural life involving contact with livestock), consumption of unpasteurized dairy products.
- Possible: possible occupational exposure (cooks or food handlers), family exposure, patients born in countries with a high prevalence of *M. bovis* infection.
- Improbable: no identified risk factors.

2.6. Statistical analysis

We compared the patient and disease characteristics of cases of TB caused by *M. bovis* and cases caused by *M. tuberculosis*. The chi-square test and Fisher exact test were used to detect differences in proportions between individuals. Additionally, we used the 1-sided Wilcoxon 2-sample rank-sum test to compare median values. Results were considered to be significant if $p < 0.05$.

3. Results

During the period 2006–2014, a total of 1586 cases of TB were diagnosed in the population studied (1,080,000 inhabitants). Of these, 1567 (98.80%) corresponded to infection by *M. tuberculosis* and 19 (1.20%) to infection by *M. bovis* (RD9, RD10 and RD11 absent in all of them). No *M. caprae* infections were found. The incidence of human infections by *M. bovis* and *M. tuberculosis* every year during the study period ranged from 0.09 to 0.46 and 14 to 20 cases per 100,000 inhabitants, respectively. The characteristics of *M. tuberculosis* and *M. bovis* according to socio-epidemiological and clinical variables are shown in Table 1. All human cases were Spaniards and were located in 4 of the 8 health districts in the province. The only characteristics that were significantly higher for infections by *M. bovis* were mortality rate (31.5% *vs* 10.5%, $p < 0.05$) and gastrointestinal tract as the primary site of disease (33.3% *vs* 5.9%, $p < 0.05$). Differences for the remaining factors were not statistically significant, although it is remarkable that there were no cases of *M. bovis* in patients younger than 24 years (0.0 *vs.* 7.8), that 13 of the 19 cases caused by *M. bovis* (68.4%) were pulmonary TB (7 of them smear-positive), and that risk factors for TB were only identified in 6 of the 19 cases caused by *M. bovis*.

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